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## BRIEF REPORT



# Anti-HLA-B7/HLA-B44 strong cross immunoreactivity observed in flow cytometry HLA-B27 immunotyping

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## Abstract

Cross reactivities are known for human leukocyte antigen inside HLA-B7 related Cross-Reactive Group (B7CREG). Some CE-IVD flow-cytometry kits use double monoclonal antibodies (mAb) to distinguish HLA-B27 and HLA-B7 but practice reveals more complexes results. This study explores the performances of this test. Analysis of 466 consecutive cases using HLA-B27 IOTest™ kit on a Navios™ cytometer from Beckman-Coulter, partially compared to their genotypes. Expected haplotypes HLA-B27-/HLA-B7- (undoubtedly HLA-B27 negative) and HLA-B27+/HLA-B7- (undoubtedly HLA-B27+) were clearly identified according to the manufacturer's instructions. On the opposite, patients strongly labeled with anti-HLA-B7 showed three different phenotypes regarding anti-HLA-B27 labeling: (1) most of the cases were poorly labeled in accordance with cross reactivity inside B7CREG (HLA-B27-/HLA-B7+ haplotype); (2) rare cases had strong B7 and B27 labeling corresponding to HLA-B27+/HLA-B7+ haplotype; (3) even less cases had strong labeling by anti-HLA-B7 but non for anti-HLA-B27, all expressing HLA-B44 and no B7CREG molecules. Surprisingly, more cases were not labeled with anti-HLA-B7 antibody but partially labeled with anti-HLA-B27 suggesting another cross reactivity out of B7CREG. mAb HLA typing suggests new, cross reactivities of anti-HLA-B27 antibody to more molecules out of B7CREG and of anti-HLA-B7 antibody but not anti-HLA-B27 to HLA-B44 molecule also out of B7CREG. HLA-B27 could surely be excluded in most samples labeled with HLA-B27, below a “grey zone” on intermediate intensity. More comparison is needed in future studies.

## KEYWORDS

B7CREG, HLA-B27 immunotyping flow cytometry HLA-B44, HLA-B7

## 1 | INTRODUCTION

Patients with previous exposure to foreign human leukocyte antigen (HLA) molecules like after pregnancy or tissue transplantation may develop an allo-immunization specific for the HLA epitopes they have been exposed to. This led to the discovery of antibodies to HLA molecules lately clarified using precise genotyping. The gold standard methods for HLA typing are historically based on micro-lympho-cytotoxicity using sensitized patient sera. More recent

methods favor immunolabeling using well characterized sera. Comparing immunological with molecular typing has revealed a lack of precision of immunological typing due to cross reactivities. Later on, immunotyping methods using monoclonal antibodies (mAb) have partly confirmed the cross reactivity and led to the definition of eplets and cross-reactive groups (CREGs) of molecules sharing common or close epitopes [1–3]. The HLA-B27 allele has been strongly associated with ankylosing spondylitis and other chronic inflammatory diseases [4–13]. HLA-B27 belongs to B7 CREG (B7CREG) that shares epitopes

with HLA-B7 and HLA-B13, B16, B17, B22, B37, B41, B42, B47, B48, B54, B55, B56, B60, B61, B73, and B81 [1, 14, 15]. HLA-B27 being part of diagnosis criteria in chronic inflammatory diseases, a rapid test has been developed using immunotyping with mAb and flow cytometry reading [16]. Few mAb are available for HLA-B27 (namely clones HLA-ABC-m3, GS145.2, and FD705). In order to rule out cross-reactivity labeling has been completed with an HLA-B7 mAb (clone BB7.1) by one kit manufacturer [17]. Flow-cytometry is a quantitative method for detection of fluorescent immunolabeling, over a large fluorescence intensity scale, making possible to differentiate intensities of labeling, resulting from densities of antigen expressed on cells and the avidity of the mAb for the antigen. Using double immunolabeling, three phenotypes were expected according to the manufacturer: (a) HLA-B7 negative/HLA-B27 negative; (b) HLA-B7 negative/HLA-B27 positive; and (c) nonconclusive HLA-B7/HLA-B27 double positive due to cross reactivity. However, daily experience shows much more phenotypes that may be difficult to conclude.

The aim of this study was to better characterize the semiology of HLA-B27 and HLA-B7 double immunotyping phenotypes.

## 2 | METHOD

### 2.1 | Samples selection

The HLA-B27/HLA-B7 immunophenotyping profiles were analyzed from 457 consecutive tests performed using IOTest™ (Beckman-Coulter, reference: A07739). The labeling was performed on ethylene diamine tetra acetic acid (EDTA)-anticoagulated whole blood followed by lysis and washing according to the manufacturer's instructions. The kit includes an anti-HLA-B27-Fluorescein (FITC; clone HLA-ABC-m3 IgG2a) and an anti-HLA-B7-Phycoerythrin (PE; clone BB7.1 IgG1), we have completed with an anti-CD45-PE-Texas red (ECD, clone J33 IgG1, reference: A07784), to focus on leukocytes. Labelings were analyzed on the Navios™ cytometer using Navios™ software (Beckman-Coulter) and under our ISO 15189 accredited process. Data analysis was performed using the Kaluza™ software (Beckman-Coulter). Labeling intensities were measured as Median Fluorescence Intensity (MdfI).

### 2.2 | Genotyping

Fifty illustrative or unexpected HLA immunotypes were compared to molecular biology which was considered as a reference method. HLA-B genotyping was performed with various molecular biology techniques depending on the initial clinical request: either Next-Generation Sequencing (NGS; Omixon® Kit Holotype 96/B) or SSO-Polymerase Chain Reaction (SSO-PCR; OneLambda® Kit locus B HD or SSP-PCR; Olerup® Kit Blow or B\*27) by the national HLA typing institute (Établissement Français du Sang Auvergne-Rhône-Alpes) according to the EFI (European Federation for Immunogenetics)

standards. HLA genotypes were expressed according to the World Health Organization (WHO) serological nomenclature (Table 1).

### 2.3 | Immunotyping

HLA-B27/HLA-B7 immunophenotyping profiles were analyzed from 466 consecutive tests performed using IOTest™ (Beckman-Coulter, reference: A07739) as described by Waeckel et al. [17]. In brief EDTA-anticoagulated whole blood was labeled using anti-HLA-B27-Fluorescein (FITC; clone HLA-ABC-m3 IgG2a) and anti-HLA-B7-Phycoerythrin (PE; clone BB7.1 IgG1), completed with an anti-CD45-PE-Texas red (ECD, clone J33 IgG1, reference: A07784), followed by lysis and washing according to the respective manufacturer's instructions. Labelings were analyzed on the Navios™ cytometer using Navios™ software (Beckman-Coulter) and under our ISO 15189 accredited process. Data were analyzed using the Kaluza™ software (Beckman-Coulter). Labeling intensities were measured as MdfI.

### 2.4 | Ethical and statistics issues

Ethical issues: the study was performed on an anonymized data base issued from routine analysis of patients in the process of diagnosis with patient approval.

Statistics were performed with Excel™ (Microsoft) and R Studio™ version 4.1.3 software and ggplot2 package. Results were expressed as mean  $\pm$  1 standard deviation and compared using Student's t-test.

## 3 | RESULTS

### 3.1 | Expected anti HLA-B27/HLA-B7 immunolabeling profiles

Analysis of a large number of routine tests evidenced the three expected profiles (Table 2):

- Two hundred and thirty-one samples (49.5%) were not labeled with anti-HLA-B7 mAb (mean value of MdfI  $1.65 \pm 0.54$  ranging from 0.1 to 3.31) and not labeled with anti-HLA-B27 mAb (MdfI:  $0.26 \pm 0.26$  ranging from 0.1 to 1.0; Figure 1A). They were concluded as not expressing HLA-B27 group according to the manufacturer's instructions.
- Seventy-four samples (15.8%) were not labeled with anti-HLA-B7 mAb (MdfI:  $1.63 \pm 0.62$ , from 0.62 to 3.59) but clearly labeled with anti-HLA-B27 mAb (MdfI:  $41.5 \pm 14.8$  from 17.1 to 93.2; Figure 1C). They were concluded as expressing HLA-B27 group.
- Sixty-two samples (13.3%) were labeled with anti-HLA-B7 mAb ( $17.8 \pm 6.2$  from 6.06 to 43) and labeled with anti-HLA-B27 mAb although at lower level ( $5.0 \pm 2.39$  from 1.34 to 10.6; Figure 1E). They were considered as non-conclusive because

**TABLE 1** Supporting Information results list of HLA-B7 and HLA-B27 genotype performed with either Next-Generation Sequencing (NGS; Omixon® Kit Holotype 96/B) or SSO-Polymerase Chain Reaction (SSO-PCR; OneLambda® Kit locus B HD or SSP-PCR; Olerup® Kit Blow or B\*27) as compared to immunotypes immunolabeling defined by IOTest™ on the Navios™ cytometer (Beckman-Coulter).

Genotype	Immunotype		
	7	indet	
8	7	indet	
8	7	indet	
13	7	indet	
14	7	indet	
15	7	indet	
15	7	indet	
17	7	indet	
18	7	indet	
18	7	indet	
18	7	indet	
18	7	indet	
27	7	indet	
35	7	neg	
35	7	indet	
35	7	indet	
	44	7	indet
	44	7	indet
	44	7	indet
	44	7	indet
	44	7	indet
	44	7	indet
	44	7	indet
49	7	indet	
51	7	indet	
51	7	indet	
51	7	indet	
51	7	indet	
51	7	indet	
52	7	indet	
56	7	indet	
60	7	indet	
40 (B61)	7	indet	
8	40	neg	
13	15	neg	
13	51	neg	
18	51	neg	
35		27	pos
38		27	pos
39		27	pos
40		27	pos
44		27	indet
49		27	pos

(Continues)

**TABLE 1** (Continued)

Genotype	Immunotype	
50	27	pos
51	27	pos
60	27	pos
	27	pos
	27	pos
	27	pos
	27	pos
	27	pos
	27	pos
49	22	neg
51	53	neg
51	60	neg
51	60	neg
51	60	neg
51	62	neg
51	62	neg
non 27		neg
non 27		neg
non 27		neg
non 27		indet
non 27		neg

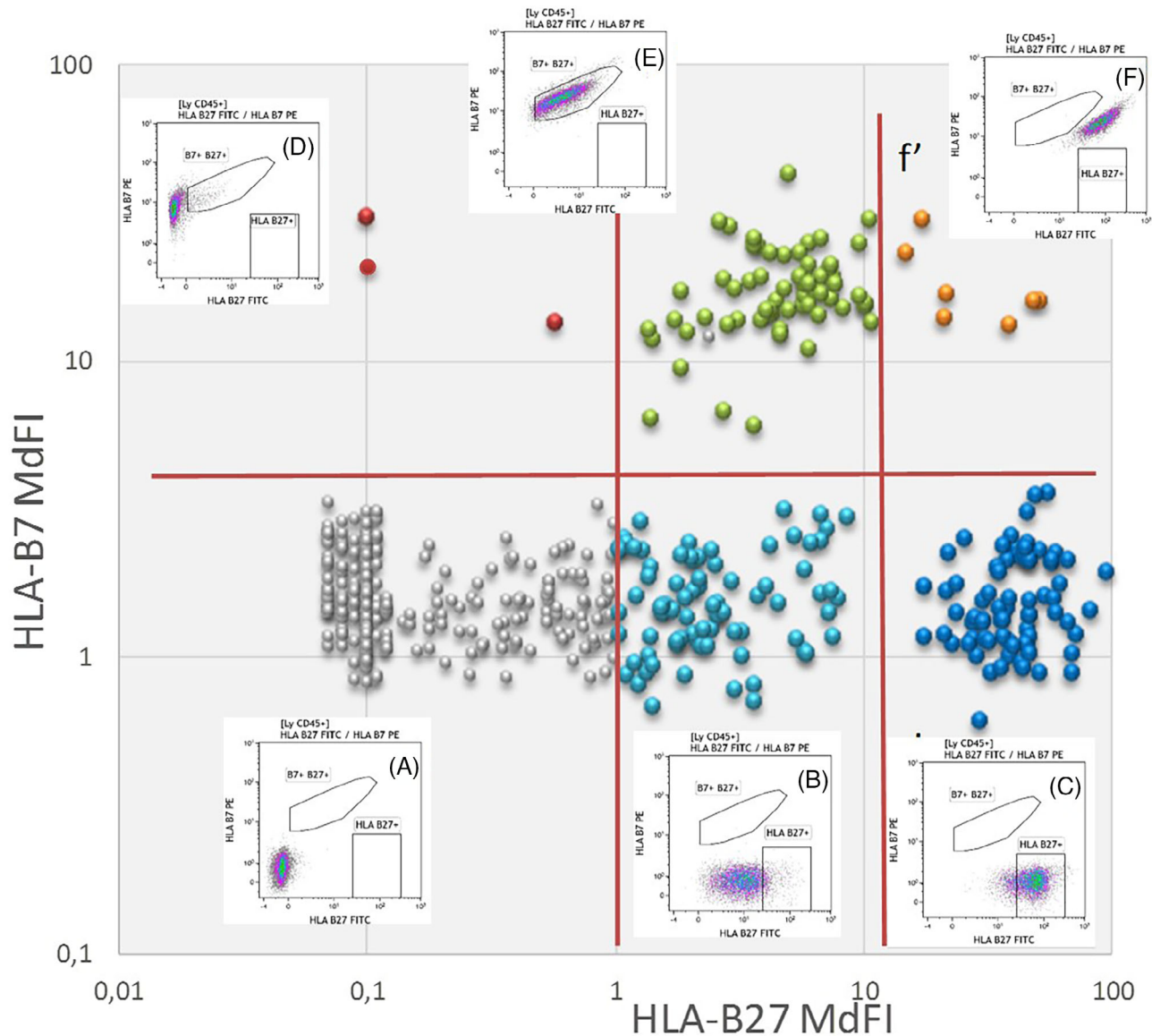
probably part of B7CREG that could provide a false positive anti-HLA-B27 labeling.

### 3.2 | However, more profiles were repeatedly observed and not explained in the manufacturer's instructions

- Eighty samples (17.1%) were not labeled with anti-HLA-B7 mAb (MdFI:  $1.64 \pm 0.61$  from 0.69 to 3.17) and were labeled with anti-HLA-B27 mAb but at lower level (MdFI:  $2.92 \pm 1.97$  from 1.02 to 8.38; Figure 1B) suggesting that anti-HLA-B27 recognized another HLA molecule with poor reactivity and not recognized by anti-HLA-B7 meaning not belonging to B7CREG.
- Seven samples (1.5%) were brightly labeled with anti-HLA-B7 mAb (MdFI:  $18.6 \pm 6.18$  from 13.2 to 30.4) and brightly with anti-HLA-B27 mAb (MdFI:  $29.8 \pm 15.0$  from 14.4 to 50.6; Figure 1F) suggesting these patients expressed both HLA-B27 and HLA-B7 molecules. This also means that the 62 samples which were strongly labeled with anti-HLA-B7 bright but dimly with anti-HLA-B27 should be considered as not expressing HLA-B27.
- Three more samples (0.6%) were clearly labeled with anti-HLA-B7 mAb ( $21.7 \pm 8.52$  from 13.5 to 30.5) but absolutely not labeled with anti-HLA-B27 mAb (MdFI:  $0.26 \pm 0.27$  from 0.10 to 0.57;

**TABLE 2** Shows respective mean ( $\pm 1$  SD) of median fluorescence intensities of different anti HLA-B7 and anti HLA-B27 immunotypes as revealed by immunolabeling with monoclonal antibodies. The % give an idea of relative frequencies out of 457 routine tests.

Profiles	Anti-HLA-B7	Anti-HLA-B27	Anti-HLA-B7 MdFI	Anti-HLA-B27 MdFI	% Observed
B27 Negative	neg	neg	1.65 $\pm$ 0.54	0.27 $\pm$ 0.30	49.5
B7-B44	pos	neg	21.7 $\pm$ 8.52	0.26 $\pm$ 0.27	0.6
B7+B27-	pos	dim	17.8 $\pm$ 6.22	5.04 $\pm$ 2.39	13.3
Bxx B27-	neg	dim	1.64 $\pm$ 0.61	2.92 $\pm$ 1.97	17.1
B7+B27+	pos	high	18.6 $\pm$ 6.18	29.8 $\pm$ 15.0	1.5
B27 Positive	neg	pos	1.63 $\pm$ 0.62	41.5 $\pm$ 14.8	15.8



**FIGURE 1** Shows six different profiles of simultaneous HLA-B27 and HLA-B7 immunotyping according to the mean fluorescence intensity of 457 tests. It also shows that cross reactivity between two B7CREG molecules is not systematic and not reciprocal. As expected, HLA-B27-/HLA-B7- cases have low MdFI (A), B27+/HLA-B7- have high HLA-B27 labeling (C), B27-/HLA-B7+ have high labeling of HLA-B7 (E) and low labeling of HLA-B27 due to cross reactivity and B27+/HLA-B7+ (F) have high double labeling. Surprisingly, numerous B27-/HLA-B7- cases show some labeling with anti-B27 (B) and rare cases show strong recognition by anti HLA-B7 antibody (D) all having HLA-B44 but not HLA-B7. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

Figure 1) as it was expected due to the known cross reactivity of anti-HLA-B27 for B7CREG molecules. This suggested that anti-HLA-B7 antibody may crossreact with another molecule out of B7CREG.

### 3.3 | Corresponding genotypes to HLA-B27/HLA-B7 immunolabeling profiles

Expected double negative and single positive results have been confirmed by genotyping on part of the samples tested. All HLA-B7+ and HLA-B27dim had the HLA-B7 genotype and not HLA-B27 except two samples that had had the two HLA-B7 and HLA-B27 genotypes and were double brightly immunolabeled by anti-HLA-B7 and anti-HLA-B27. On the other hand, none of the HLA-B7 negative and HLA-B27 dim samples had HLA-B7 or HLA-B27 genotypes. Few of them had one genotype from the B7CREG (HLA-B13; HLA-B56; HLA-B60) but few more, out of B7CREG, suggesting anti-HLA-B27 mAb (and not anti-HLA-B7 mAb) was cross-reacting with other HLA-B molecules out of the B7CREG. More interestingly, eight patients had then HLA-B44 genotype. Surprisingly, six out of them, had HLA-B44 and HLA-B7 genotypes and were brightly labeled by anti-HLA-B7 and dimly by anti-HLA-B27 mAb. However, two patients had HLA-B44 but not HLA-B7 genotype and were the one brightly labeled with anti-HLA-B7 and not at all with anti-HLA-B27 mAb.

### 3.4 | Anti HLA-B27/HLA-B7 cross immunoreactivity is not reciprocal

When the genotypes were known, we could observe that HLA-B7 immunolabeling fluorescence MdFI were not increased on samples co-expressing B7/B27 ( $10.8, n = 1$ ) or B7/another B7CREG molecules (HLA-B13; HLA-B56; HLA-B60;  $17.7 \pm 2.97; n = 3$ ) or B7/B44 ( $15.1 \pm 4.76; n = 6$ ) as compared to HLA-B7 without one of these B7CREG molecules ( $18.7 \pm 6.6, n = 12$ ). On the other hand, HLA-B7 immunolabeling MdFI were not different when HLA-B27 was positive ( $1.31 \pm 0.89, n = 19$ ) or dim with B7CREG ( $1.84, n = 1$  or not) ( $1.17 \pm 0.45, n = 9$ ) compared to HLA-B27 negative ( $1.56 \pm 0.28, n = 9$ ).

Similarly, HLA-B27 immunolabeling MdFI were widely distributed ( $41.8 \pm 30.5, n = 19$ ), being at the lower limit even in one case with HLA-B27 co-expressed with HLA-B7 (MdFI:  $12.5, n = 1$ ). Surprisingly, HLA-B27 MdFI was extremely strong in one another case with co-expression of HLA-B27 and HLA-B60, a B7CREG (MdFI =  $62.4$ ). On the other hand, HLA-B27 partial cross-reactivity with dim labeling were at similar MdFI when the labeling was due to cross reactivity to HLA-B7 ( $7.20 \pm 3.21, n = 3$ ) alone or with another B7CREG ( $7.75 \pm 5.78, n = 3$ ). It tended to be lower when cross reactivity was due not due to HLA-B7 but to another B7CREG ( $2.93, n = 1$ ) or to molecules out of B7CREG ( $5.81 \pm 4.29, n = 9$ ), still significantly higher than double negative samples ( $0.35 \pm 0.30, n = 9; p = 0.005$ ).

## 4 | DISCUSSION

Beside the well-expected double negative and HLA-B27 positive labeling that could be easily concluded for diagnosis, unexpected results have raised new questions. First, some HLA-B27 dim cases were clearly positive for HLA-B7, in accordance with the genotyping. Indeed, HLA-B7 is part of HLA-B27 related Eplet 80N: B\*27:08 (super type Bw6). Cross reactivity was expected, and the labeling was at much lower fluorescence intensity due to a lower affinity of anti-HLA-B27 mAb to HLA-B7 as compared to HLA-B27 molecules. On the opposite, anti-HLA-B7 mAb did not detect HLA-B27 molecules and anti-HLA-B7 immunolabeling was not enhanced in case of HLA-B7/B27 co-expression, suggesting that anti-HLA-B7 mAb did not cross-react with HLA-B27 molecule meaning that cross-reactivities inside B7CREG are not reciprocal with these mAb.

On the other hand, we observed a new type of cross reactivity leading to a strong labeling with anti-HLA-B7 mAb but not with anti-HLA-B27 mAb. The cases are rare and genotyping had shown that all of these observed cases had HLA-B44 genotype but no genotype from the B7CREG. HLA-B44 was not very rare in the population we have tested but was frequently associated with HLA-B7, explaining why this effect was not easily observed. This suggests that anti-HLA-B7 mAb strongly crossreact with HLA-B44 molecules but not anti-HLA-B27 mAb although at least some eplets of HLA-B44 are part of HLA-B27 related Eplet 82LR group: B\*27:03/05 (super type Bw4). Unfortunately, the genotyping method we used did not differentiate between Bw4 and Bw6 as NGS would have done.

The second main point we observed is that anti-HLA-B27 mAb was partially detecting samples of B7CREG but also more molecules, not recognized by anti HLA-B7 mAb and most of the time out of the B7CREG.

The physio-pathological significance of these crossreactivities is not known. The association of HLA-B27 with some chronic inflammatory diseases may be related to the capacity for the patient's T cells to recognize some pathogenic HLA-B27-peptide complexes. As the TCR recognize the epitope in a similar way as antibodies, this raises the possibility that other, rare HLA B groups can mislead T cells recognition and eventually favor the same diseases. This remains to be ruled out.

On a practical point of view, concerning the interpretation of the test, anti-HLA-B7 labeling is not sufficient to exclude risks of cross reactivity of anti HLA-B27 mAb. Furthermore, Anti HLA-B27 labeling intensity is critical but strong or dim immunolabelings have a small overlap also called "grey zone" in which it is difficult to conclude with high degree of certitude the HLA-B27 as discussed somewhere else [17]. The large discrepancy in immunolabeling also suggests that all HLA-B27 and other molecules are not equally recognized by the same mAb, possibly due to minor genotypic polymorphisms or some post translational processing like glycosylation.

Last remark, our results confirm that anti-HLA-B7 mAb has poor cross reactivities as discussed before and HLA-B27 can be reasonably excluded in cases of anti-HLA-B7bright but anti-HLA B27 dim

phenotype proposed to be concluded as “non-conclusive” by the manufacturer. On the other hand, HLA-B27 is certain when both labelings are bright, out of the grey zone.

In conclusion, analysis of large quantity of HLA-B27/HLA-B7 immunotyping led us to describe multiple phenotypes and has shown that the manufacturer instructions are too simplistic and sometime misleading. While the aim of this tests is to avoid costly and time-consuming genotyping to detect with highest probability, patients with HLA-B27 group. Manufacturer instructions say that only HLA-B27+HLA-B7 negative haplotypes can be concluded. Our results show that strong anti-HLA-B27 labeling can be considered as HLA-B27 even in cases of anti-B7 labeling (typing double HLA-B7+/HLA-B27+ haplotype) provided a grey zone is defined. Thus, most HLA-B27 dim HLA-B7+ cases, classified as “non-conclusive” can firmly excluded HLA-B27. We also show that anti-HLA-B7 antibody has some cross reactivity with HLA-B44 and anti-HLA-B27 antibody has some cross reactivities with other genotypes all out of the B7CREG paradigm. More comparison is needed in future studies.

## AUTHOR CONTRIBUTIONS

**Fabien Francois:** Investigation; validation; formal analysis; writing – review and editing; writing – original draft. **Louis Waeckel:** Investigation; validation; formal analysis; writing – original draft; writing – review and editing. **Anne-Emmanuelle Berger:** Investigation; writing – review and editing. **Claude Lambert:** Conceptualization; investigation; validation; formal analysis; writing – original draft; writing – review and editing; project administration.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/cyto.a.24824>.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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